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Alteration of Microflora of the Facultative Parasitic Nematode Pristionchus Entomophagus and its Potential Application as a Biological Control Agent

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ALTERATION OF MICROFLORA OF THE FACULTATIVE PARASITIC NEMATODE *PRISTIONCHUS ENTOMOPHAGUS* AND ITS POTENTIAL APPLICATION AS A BIOLOGICAL CONTROL AGENT

by

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Abstract:

Pristionchus entomophagus is a microbivorous, facultative, parasitic nematode commonly found in soil and decaying organic matter in North America and Europe. This nematode can form an alternative juvenile life stage capable of infecting an insect host. The microflora of *P. entomophagus* is highly variable and may contribute to insect host mortality. *Pristionchus entomophagus* is not associated with specific bacterial species, and its microflora may possibly vary with habitat and/or hosts. The goal of this project was to develop protocols to transfer labeled bacteria to *P. entomophagus*, and then assess transfer of the labeled bacteria to an insect host via exposure to altered nematodes. Successful inoculation of the nematodes and transfer to the insect would provide a method to enhance virulence of *P. entomophagus* for use against *M. rubra* and other pest insects. I successfully developed a protocol to alter the microflora of *P. entomophagus* nematodes, but was unable to confirm transfer of the target bacteria by nematodes to *Galleria mellonella* larvae. The internal microflora of most adult nematodes was altered and juveniles exposed to the labeled bacteria were seen to carry it on the external surface of their cuticle. Juveniles are the infective stage, and without internal haborage, they may be poor vectors of the target bacteria. Another challenge in developing *P. entomophagus* as a biological control agent is potential nematicidal activity of entomopathogenic bacteria. Overall, the results of this study are promising and provide the initial steps towards the development of *P. entomophagus* with enhanced virulence as a biological control agent.

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Introduction:

General Nematode Biology and Diversity

Phylum Nematoda, commonly referred to as roundworms, are among the most genetically diverse and abundant multicellular organisms (Raven 1998). Though fewer than 25,000 species have been described, there are an estimated 500,000 to 100,000,000 species worldwide. Diverse morphological, behavioral, and physiological adaptations have allowed nematodes to adapt to virtually every habitat including 75% that are free-living in aquatic and soil habitats (Roberts 2000). Most are microbivorous (consume bacteria, fungi, and/or algae), while others are herbivorous, predaceous, or parasitic (hosts include plants, invertebrates or vertebrates) (Raven 1998).

Insect- Nematode Interactions

It is estimated that 90% of insect pests have at least one developmental stage in soil (Akhurst 1986), providing ample opportunity for interactions between nematodes and insects. More than thirty nematode families are known to associate with or parasitize insects (Stock and Hunt 2005). Common insect-nematode interactions include phoretic, necromenic, facultative parasitic and obligate parasitic. Potentially the most common interaction (at least 14 nematode families including Diplogasteridae) is phoretic (Poinar 1975). Phoretic and necromenic interactions may be a pre-adaptation to parasitic insect-nematode interactions (Dillman et al. 2012).

In phoretic interactions the insect serves only as a means of transport and dispersal and is not necessary for the survival of the nematode (Poinar 1975). Dauer stage nematodes are free-living, highly resistant to unfavorable environmental conditions,

non-reproductive infective juveniles (Bellows 1999). The dauer stage is an alternative third instar juvenile stage that retains the cuticle of the second instar. Nematodes typically enter the dauer stage when environmental conditions are unfavorable and during phoretic life stages. In phoretic interactions, dauer juveniles typically attach to internal or external surfaces of the insect and rarely have a negative impact upon the host (Poinar 1975).

Another type of insect-nematode interaction is necromenic association, a more specific interaction than phoretic in which nematodes enter the host insect and feed on bacteria and fungi that proliferate upon host death (Rae et al. 2008). Nematodes in the genus *Pristionchus* are often considered to have necromenic interactions with their insect hosts. Necromenic nematodes may be unable to parasitize and utilize host nutrients until the host has died and therefore are unable to mature and reproduce in the living host (Poinar 1972). Necromenic insect interactions may be a pre-adaptation to parasitism as the nematode is exposed to low oxygen and toxic host enzymes (Rae et al. 2008). The genome of *Pristionchus pacificus* (Diplogasterida: Diplogasteridae Sommer), a necromenic nematode, has abundant genes encoding for degradative and detoxifiying enzymes, which assist in survival in the harsh and toxic insect cadavers (Dieterich et al. 2008)

Parasitic interactions include both obligate (no free-living stage and requires a living host to complete its lifecycle) and facultative parasites (gains nutrients from host and are capable of reproduction in the absence of an insect-host environment) (Rae et al. 2008, Poinar 1972). The damage caused by both facultative and obligate parasites varies tremendously and does not necessarily result in host death. Facultative parasites are

found in a variety of nematode families, including: Diplogasteridae, Tylenchida, Neotylenchidae, and Rhabditidae. The mechanism of facultative parasitism can vary between species. *Pristionchus aerivora* (Rhabditida: Diplogasteridae Cobb) can be considered a facultative parasitic nematode because it requires an insect host for the maturation of juvenile nematodes but not for reproduction of the adults. In *P. aerivora*, adult nematodes exit and reproduce outside of the host (Chitwood 1974). Many diplogasterid nematodes that mature and reproduce in insect hosts in the wild can mature and reproduce without insect hosts in the laboratory (Poinar 1972). Facultative parasitic Diplogasterid nematodes typically lack highly specific nematode-bacteria interactions. It is especially important to note that due to a lack of grinder in the terminal bulb of the pharynx (present in Rhabditids); Diplogastrid nematodes do not fully lyse bacteria and can disseminate viable bacteria that can contribute to insect mortality (Rae et al. 2008).

Obligate insect parasites can be found in at least 13 nematode families, with the best understood examples being in the orders Mermithidae, Steinernematidae, and Heterorhabditidae (Bellows 1999; Poinar 1975). Most obligate parasitic nematodes parasitize only one host during development, with only three families utilizing multiple hosts. *Steinernema* and *Heterorhabditis* nematode species have symbiotic interactions with the bacteria genera *Xenorhabdus* and *Photorhabdus,* respectively. This symbiosis between bacteria and nematode is essential for bacteria and nematode survival, as well as host death (Rae et al 2008). Daeur juveniles of these species actively seek and enter their hosts, where they release pathogenic symbiotic bacteria (Bellows 1999). Juveniles mature and reproduce within their cadaver, and reassociate with symbiotic bacteria before emerging from the cadaver (Dillman et al. 2012). Symbiotic bacteria does not appear to

be important in Mermithidae nematode pathogenesis, as nematodes mature to third instar juveniles within their host and cause host mortality by puncturing the hosts' cuticle as it exits (Bellows 1999).

Entomopathogenic Nematodes as Biological Control Agents

Nematodes are frequently associated with insects, mites, and molluscs of importance to agricultural, health, and forest industry (Stock and Hunt 2005). Of the more than 30 nematode families associated with insects, only seven have been major targets of biological control research, including: Mermithidae, Allantonematidae, Neotylenchidae, Sphaerularidae, Rhabditidae, Steinernematidae, and Heterorhabditidae, with the latter two being the main focus for biological control programs (Lacey et al. 2001). Although these seven families have been the focus of nematode biological control research, other nematode families have the potential to be utilized as biological control agents (Stock and Hunt 2005).

Nematodes utilized for biological control are almost always obligate parasites, with *Deladenus siricidicola* (Tylenchida: Neotylenchidae Bedding) being the notable exception as it can alternate between parasitic and mycetophagus life cycles (Stock 2005). Parasitic nematodes are considered true entomopathogenic nematodes when they have a symbiotic relationship with pathogenic bacteria that facilitates rapid mortality relative to other types of insect-nematode associations and are unambiguously distinguishable from phoretic, necromenic, and other parasitic interactions (Dilman et al. 2012).

Nematodes of the family Mermithidae are obligate parasites and are among the largest nematodes (up to 20 cm) that associate with insects (Bellows 1999).

Romanomermis culicivorax (Mermithida: Mermithidae L.) parasitizes mosquitos and was briefly investigated as a potential biological control agent for mosquito control. However, this research was mostly discontinued because of high cost, relatively poor efficiency, and the advent of commercially available *Bacillus thuringiensis* (Bellows 1999, Stock 2005). Due to various aspects of Mermithid life history, use of Mermithids as biological control agents result in only moderately long term reduction in insect populations (Stock 2005). The Tylenchid nematode, *Deladenus siricidicola*, was successfully used against the European wood wasp (*Sirex noctilio* (Siricoidea: Siricidae Fabricius)), an invasive wasp introduced into Australia from imported exotic pine trees. Due to the peculiar life cycle of *D. siricidicola* (a parasitic and a mycetophagous life cycle), it can also be mass produced, an important quality in commercializing biological control agents.

Entomopathogenic nematode research has focused upon the families Steinernematidae and Heterorhabditidae and is currently a very active field of research (Stock 2005). These nematodes have mutualistic association with pathogenic bacteria genera (Bellows 1999). *Xenorhabdus*, the symbiotic bacteria of *Steinernema* nematodes, and *Photorhabdus*, the symbiotic bacteria of *Heterorhabditis* nematodes, have a complex life cycle that includes a symbiotic phase within the nematode and a pathogenic phase within the insect host (Forst 1995). The nematodes require the presence of the symbiotic bacteria within the cadaver for reproduction (Gaugler 2002). Free living isolates of *Xenorhabdus* and *Photorhabdus* have not been isolated from soil or water, and may be unable to survive in the natural environment without symbiotic association with nematodes (Forst 1995). The pathogenicity of specific *Xenorhabdus* and *Steinernema* species varies. For example *X. nematophilus* is highly pathogenic to the tobacco horn

worm (*Manduca sexta* (Lepitodoptera: Sphingidae L.)), an organism inherently with strong defenses against pathogenic bacteria, while *X. poinarii* and *X. japonica* appear to lack pathogenic factors present in other *Xenorhabdus* species (Forst 1995).

Upon accessing the hemocoel through the spiracles or directly through the cuticle, the nematode releases bacteria into the host's hemolymph where the bacteria proliferate (Snyder et al. 2007). In Steinernematids, *Xenorhabdus* bacteria are released through the anus. After release, the bacteria secrete several products, including: lipase, phospholipase, protease, and antimicrobial compounds (Bellow 1999). These degradative enzymes break down macromolecules within the insect, providing nutrients to the reproducing nematodes while the antimicrobial compounds suppress the growth of other microorganisms in the cadaver. When nematode populations are high and nutrients become limiting, the nematodes reassociate with the symbiotic bacteria, and exit the cadaver to seek a new host (Snyder et al. 2007). Colonization of symbiotic bacteria in the nematodes occurs in a specialized vesicle, a distended region of the anterior portion of the intestine. In the vesicle, one or a few bacterial cells bind to an unattached acellular mass (subcellular intravesicular structure) and proliferate until the vesicle is full (Snyder et al. 2007).

A few genera of Diplogasterid nematodes have been studied as plant biological control agents (Stock and Hunt 2005). The ease of *in vitro* culture, short life cycle, high rate of predation, ability to switch to bacteriophagy in the absence of prey, and an environmentally resistant daeur stage make Diplogasterid nematodes strong candidates for use as biological control agents. Diplogasterid nematodes frequently have phoretic and necromenic interactions, and occasionally facultative parasitic interactions with

insects (Poinar 1975). With various morphological and behavioral adaptations being amenable to use as biological agents and frequent interactions with insects, Diplogasterid nematodes are potentially favorable targets of biological control research. In addition to these important qualifications, Diplogasterid individuals also lack a grinder in their terminal bulb and therefore are able to disseminate intact bacteria (Poinar 1975, Chantanao and Jensen 1969).

Phylum: Nematoda, Order: Diplogasterida, Family: Diplogasteridae

The family Diplogasteridae is a cosmopolitan nematode family commonly found feeding upon bacteria and other microrganisms in soil and decomposing organic matter (Poinar 1975). Diplogasteridae is monophyletic group within the Rhabditidae and includes 300 known free-living species in 28 genera. Most diplogastrids are gonochoristic (unisexual), with hermaphrodites found only in the genera *Pristionchus* and *Diplogasteroides* (Mayer 2007).

A peculiar feature of Diplogastrid development, including *Pristionchus*, is three postembryonic juvenile stages where most nematodes have four (Fürst von Lieven 2005). The first and second postembryonic molts occur before hatching and the nematode hatches as a second instar juvenile instead of a first instar juvenile (Fürst von Lieven 2005; Hong 2006). As a result of the first juvenile instar molting inside the egg, the mouthparts (stoma) have more time to develop a higher degree of complexity (found in 75% of described Diplogastrids) including; movable teeth, claws, and denticles. Complex stoma morphology is used to feed on fungal spores, ciliates, and other small metazoans. Though these food sources are present in niches where Rhabditidae are

present, only Diplogastrids evolved mechanisms to access these potential food sources. Another unique morphology is a terminal bulb (typical for Rhabditids) that lacks a grinder (unique to Diplogastrids) (Fürst von Lieven and Sudhaus 2000).

Juvenile stages frequently exhibit phoretic (internal and external) interactions with beetles, termites and ants (Poinar 1975). Other Diplogasterid species can act as facultative parasites (including *Pristionchus uniformis* in the potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae Say) in which daeur juveniles rapidly proliferate in the host's intestine causing mortality before feeding and multiplying in the cadaver (Poinar 1975).

Genus: *Pristionchus*

Pristionchus is a genus of nematode with a cosmopolitan distribution and diversity of insect hosts. *Pristionchus* nematodes have been collected directly from insects (predominately beetles), from soil, and decaying organic matter (Sudhaus and Furst von Lieven, 2003, Herrmann et al. 2006a). The 31 described and several undescribed species of *Pristionchus* have diverse biogeographic distributions and reproductive strategies (gonochoristic or hermaphroditic) (Mayer et al. 2007, Herrmann et al. 2006).

Nematodes of the genus *Pristionchus*, particularly *Pristionchus pacificus*, have been utilized as model organisms due to their high brood size, short generation time, and hermaphroditic reproduction (Hong 2006). Diplogasteridae (including *Pristionchus* pacificus) is a sister group of some rhabidids (including *C. elegans*) based upon molecular evidence (Blaxter 1998). Due to the relatedness to *C. elegans*, *P. pacificus*

was originally studied as a comparative model organism for *C. elegans* and later as model organism for developmental and evolutionary biology. *Pristionchus pacificus* was the first non-*Caenorhabditis* nematode with a fully sequenced genome (Mayer et al. 2007, Hong 2006). Unlike *C. elegans* and like all nematodes of the family Diplogasteridae, *Pristionchus* lacks a grinder in the terminal bulb which normally disrupts and often lyses ingested bacteria (Rae et al. 2008). As *Pristionchus* nematodes lack a grinder, they are capable of disseminating whole and viable bacteria (Rae et al. 2008, Chantanao and Jensen 1969). A study found *P. lheriteiri* capable of ingesting and dispersing viable plant pathogenic bacteria and fungi. *P. lheriteiri* and other saprozoic (consumption of decaying organic matter) nematodes have a significant role in dissemination and survival of bacterial pathogens (Chantanao and Jensen 1968).

Pristionchus-insect interactions are a recent area of research beginning less than ten years ago (Herrmann, M et al. 2006, Rae et al. 2008). *Pristionchus* nematodes are mostly associated with beetles, including: scarab beetles (Scarabaeidae ssp), Colorado potato beetles (*Leptinotarsa decemlineata*), and oriental beetles (*Exomala orientalis*). *Pristionchus pacificus* was found on the oriental beetle in Japan and the United States (Mayer et al. 2007). *Pristionchus maupasi* and *P. entomophagus* are predominately found on cockchafers (*Melolontha* ssp.) and dung beetles (*Geotrupes* ssp.).

Pristionchus nematodes are not associated with specific bacteria species and are capable of ingesting a diversity of bacteria (Rae et al. 2008). Bacteria isolated from *P. entomophagus* and *P. lheriteiri* nematodes from German soil consisted mainly of bacteria in the orders Pseudomonadales, Burkholderiales, Flavobacteriales, and Xanthomonadales as well as plant pathogens (*Agrobacterium and Erwinia)*, and human

pathogens (*Bordetella*, *Burkholderia*, and *Microbacterium*) (Rae et al. 2008).

Pristionchus entomphagus is hermaphroditic and found in Europe and North America (Mayer et al. 2007). It has been suggested that it may have been introduced to North America from Europe by beetles and/or human activity. *Pristionchus entomophagous* has been found in association with dung beetles, June beetles (Herrmann et al. 2006, Mayer et al. 2007), and the European fire ant (*Myrmica rubra*) (Groden et al. 2010). *Pristionchus entomophagous* was more attracted to dung beetle bacteria than *P. maupausi* and *P.* pacificus (Rae et al. 2008). Dumont (2011) examined the bacteria associated with *P. entomophagus* originally isolated from *M. rubra.* The bacteria species that he found on the cuticle and in the gut of *P. entomophagus* and hemolymph of infected *Galleria mellonella* include species that are associated with insects and soil (including rhizosphere of plants and fungi). Bacteria identified from the digestive tract included various *Paenibacillus spp*, and bacteria identified from hemolymph *Galleria mellonella* larvae infected with the nematodes included; *Serratia marcescens, S. nematodiphila*, *Pseudomonas fluorescens*, and *Delftia spp.* In Germany, Rae et al. (2008) isolated bacteria from *P. entomophagus* collected from Scarab beetles (*Geotrupes spp.*), identifying *Serratia spp*., *Pseudomonas spp.* (including *P. filiscindens*), *Bacillus spp.*, *Ochrobactrum* sp. *Proteus Vulgaris*, and *Enterobacter amnigenus.*

<u>*Pristionchus* nematodes and their potential as biological control agents</u>

Nematodes in the genus *Pristionchus* consume a variety of bacterial species (Rae et al. 2008) and *P. lheritieri* thrive upon *Serratia marcescens* and several pathogenic phytobacteria species in laboratory cultures (Chantanao and Jensen 1968). As

Pristionchus nematodes have no grinder in the terminal bulb of their pharynx, they are able to disseminate viable bacteria in the environment through defecation and adherence of bacteria to their cuticle. Nematodes are major components of the soil ecosystem and *Pristionchus* nematodes may have a significant role in dissemination of bacteria in soil ecosystems (Chantanao and Jensen 1968, Rae et al. 2008, Wei et al., 2003). *Pristionchus entomophagus* (Diplogasterida: Diplogasteridae Kreis) containing entomopathogenic bacteria internally and/or externally may be capable of disseminating entompathogenic bacteria to insect hosts (Rae et al. 2008) and could potentially be utilized as biological control agents.

Pristionchus entomophagus demonstrates low bacterial species specificity in the microflora. It is associated with and attracted to a variety of bacterial species (including entomopathogenic bacteria) such as; *Pseudomonas* spp, *Serratia* spp., *Enterobacter* spp. and *Bacillus* spp. (Rae et al 2008). *Pseudomonas fluorescens,* found in association with *P. entomophagus* (Dumont 2011), produces hydrogen cyanide and has demonstrated the ability to cause insect mortality by inhibiting cytochrome *C* oxidase in the respiratory chain of the termite *Odontotermes obesus* (Isoptera: Temitidae Ramb.) (Devi and Kothamsi 2009, Rae et al. 2008). *Serratia marcescens,* again found in association with *P. entomphagus* (Dumont 2011), demonstrated highly insecticidal activity when exposed to cutworms (*Spodoptera litura* (Lepidoptera: Noctuidae Fabricius) (Rae et al. 2008, Nishiwaki et al. 2007). Though insect susceptibility to *P. fluorescens* and *S. marcesans* is variable, the addition of the aforementioned bacteria and other entomopathogenic bacteria to *P. entomophagus* may enhance the virulence of the nematode.

Not all bacteria with insecticidal activity have the potential to be transferred to

insect hosts by *Pristionchus* nematodes, as nematodes can recognize and avoid some entomopathogenic bacteria that negatively impact their fitness. *Pristionchus* nematodes, including *P. entomophagus*, demonstrated a strongly adverse response in chemotaxis assays to *Bacillus thuringiensis*, a bacterial species that produces insecticidal crystal proteins (ICP) that can be toxic to nematodes (Frankenhuyzen 2009, Rae et al. 2008, Wei et al. 2003). Other *Bacillus* species have nematicidal as well as insecticidal activity, and can reduce fecundity in *Pristionchus* spp. (Rae et al. 2010). *Pristionchus maupasi* and *P. pacificus* demonstrated only weak positive chemotactic response to the bacteria *Serratia* spp., *Ochrobactrum* spp., *Enterobacter* spp. and *P. vulgaris*. Though these bacterial species are commonly found in *Pristionchus* nematodes, the nematodes may not prefer them as a nutritional source and sometimes even avoid them.

However, the same study demonstrated that *P. pacificus* is more resistant to *Bacillus* spp. and pathogenic bacteria in general in comparison to *C. elegans* (Wei et al. 2003, Rae et al. 2010). *Pristionchus pacificus* has an abundance of genes encoding for detoxification and degradation enzymes, which is consistent with necromenic nematode-insect interactions (Dieterich et al. 2008). Unlike *C. elegans*, *Pristionchus* nematodes are necromenic and proliferate inside the harsh environment of insect cadavers with potentially toxic soil bacteria (e.g. *Bacillus* ssp.) (Rae et al. 2008, Dieterich et al. 2008). Because of this, *Pristionchus* nematodes likely needed to evolve recognition of nematocidal bacteria and detoxification mechanisms to survive in the harsh environment of insect cadavers. *Pristionchus* nematodes are also capable of suppressing spore germination in their intestine and using pathogenic spore forming (including *Bacillus* spp.) and non-spore forming pathogenic bacteria (*P. aeruginosa*, *P. fluorescens* and

Staphylococcus aureus) as a food source (Rae et al. 2008). *Pristionchus* nematodes are susceptible to insecticidal crystal proteins (ICP) produced by *B. thuringiensis* and produce smaller brood sizes when exposed to ICP (Rae et al. 2010, Wei et al. 2003). However, as *Pristionchus* nematodes lack a grinder, they are also rarely directly exposed to the insecticidal crystal proteins and functionally can be resistant to ICP. The generally strong resistance of *Pristionchus* spp. to pathogenic bacteria and their pharynx morphology makes them good candidates to transfer viable insecticidal bacteria to insect hosts and act as biological control agents.

This project is investigating the biological control potential of *Pristionchus* nematodes against the invasive European Fire Ant (*Myrmica rubra* (Hymenoptera: Formicidae L.)). In 2003 & 2008 *Pristionchus entomophagus* was found emerging from *M. rubra* collected from several sites in Maine (Groden et al. 2010). Specifically, this project is determining the protocol for transferring specific bacteria to *Pristionchus* nematodes and ultimately to their insect host.

Ants and Entomopathogenic Nematodes

Myrmica rubra is a palearctic northern temperate ant species with a native range located as far north as the Arctic Circle and as far south as the Black Sea and is found throughout the United Kingdom, northern Europe and western Siberia (Groden et al. 2005). *Myrmica rubra* was unintentionally introduced to North America in the early 20th century and has since become a prolific and deleterious invasive species. Museum records suggest that *M. rubra* was likely introduced on multiple occasions through the transport of plant materials by plant nursery industries. *Myrmica rubra* is currently found in eastern Canada and the Northeastern United States, including: Maine (particularly

coastal regions and Acadia National Park), Massachusetts, New Hampshire, New York, Ontario, Rhode Island, and Quebec, and the maritime provinces (Groden et al. 2005). More recent records indicate populations on the west coast in Vancouver, British Columbia (Higgins, pers. Comm. 2013), and Seattle, WA (Wetterer and Radchenko 2010).

Habitats where *M. rubra* are found are often very moist and include deciduous forests, old fields, lawns and gardens, shorelines, and wetlands. Nests in the northeastern USA are typically found in leaf litter, woody debris, decaying logs, and human debris (Groden 2004). Exotic species such as *M. rubra* can dominate the habitat and outcompete native species for resources (Ouellette 2010). Nest densities in these introduced regions tend to be unusually high and the painful sting inflicted by these ants affects native biodiversity and homeowners' use of property (Groden et al. 2005). In sites in Acadia National Park where *M. rubra* is present the densities of native ant species (including 42 species represented by 15 genera and 5 subfamilies) significantly declines and are frequently absent (Garnas 2005, Oullette 2010).

Attempts to limit the spread of and control *M.* rubra populations have had little long-term success (Groden 2004). Conventional insecticides have been largely ineffective against *M. rubra* for long-term control, as populations often rebound after treatment. A potential method of controlling *M. rubra* populations is through the use of biological control agents including parasitic nematodes (Morris 1990).

Ants are parasitized by several nematode families, including: Mermithidae, Tetradonematidae, Allantonematidae, Seuratidae, Physalopteridae, Steinernematidae, Heterorhabditidae, Rhabditidae, Diplogastridae, and Panagrolamidae (Poinar 2012). Ants

have long been parasitized by nematodes, with the earliest known example found within 40 million year old Baltic amber containing a male ant with an emerging juvenile nematode from the order Mermithidae (Poinar 2002). Ant-nematode interactions are diverse and include indirect parasitism by Mermithid nematodes (eggs are the infective stage rather than larvae), internal and external phoresis in Diplogastrid nematodes, and true entomopathogenic parasitism by Steinernematid and Heterorhabditid nematodes.

In 2003 & 2008 a diplogasterid-like nematode was found emerging from *M. rubra* collected from several sites in Maine and identified as *Pristionchus entomophagus* (Groden et al. 2010). *Pristionchus entomophagus* is readily capable of infecting and causing mortality in *M. rubra* facilitated by bacteria found in the gut and on the cuticle of the nematode (Rae et al 2008). As *P. entomophagus* is not associated with specific bacterial species, selected insect pathogenic bacteria could hypothetically be transferred to *P. entomphagus* nematodes. *Pristionchus entomophagus* nematodes with artificially enhanced virulence potentially may cause higher mortality than unaltered *P. entomophagus* in insect pests including *M. rubra*.

Materials and Methods:

Harvesting and storing *Pristionchus entomophagus*

Myrmica rubra colonies were collected from sites in Acadia National Park and Orono, ME where *Pristionchus entomophagus* infection has previously been confirmed. Approximately 200 specimens from each of sixteen *M. rubra* colonies were collected with manual aspirators from each of eight sites in September 2012 (Table 1). Colonies were held at ambient temperature (approximately 21° C) in 10 x 14 cm^2 plastic nest boxes with vented lids. Ants were fed ca. 2 g of sugar and tuna diet mix every 2-4 days, and provided with water on a 3x3 cm^2 piece of sponge. Colonies were checked every other day and dead ants were removed with sterile forceps. Ant cadavers were surface sterilized by submersion in 0.1% zephiran chloride solution followed by two rinses in de-ionized (DI) water. Each surface sterilized cadaver was stored in an individual well of a 48 well plate which was misted with DI water. Ants from different colonies were stored in separate plates and were maintained at 21° C. To maintain high humidity, nest boxes were placed inside plastic bags containing damp paper towels. Cadavers were monitored for nematode emergence every other day. As nematodes emerged, they were pipetted from the wells and transferred to 50 mL centrifuge tubes with DI water and held at 10° C.

Table 1: GPS coordinates of *Myrmica rubra* colony collection sites.

Mount Desert Island, Maine	
Woodchip	68°15'23"W 44°22'37"N
Otter Cliff Road	68°12'1"W 44°19'45"N
Visitors Center	68°14'53"W 44°24'37"N
Sports Park	68°12'12"W 44°22'52"N
College of the Atlantic	68°13'21"W 44°23'41"N
Old Farm Road	68°11'42"W 44°22'24"N
Breakneck Road	68°15'22"W 44°22'39"N
Orono, Maine	
Orono	68°40'3"W 44°53'14"N

Virulence of Nematode Populations

Assays were conducted to determine if virulence varied among nematode populations as was observed in previous studies. Waxworm larvae (*Galleria mellonella*) were exposed to the different populations of nematodes and monitored for mortality. In separate assays for each nematode population, *G. mellonella* larvae were exposed to low (20-25) and high (200-250) dose treatments of nematodes and compared to a control treatment with no nematodes. Four replicate plates with 5 waxworms per plate were exposed per treatment in each assay. The waxworm larvae were placed in 100×20 mm^2 petri dishes containing 20 g of autoclaved sand moistened with 5 ml of DI water. Nematode in DI water solution were added to the sand, dishes were held at ambient temperature (approximately 21° C), and larvae were observed for mortality daily for 14 days. The sand in the dishes was remoistened as needed. Survival analysis was conducted using a general parametric model based on the Weibull distribution to examine difference in time to death between sites and nematode treatments (JMP, SAS Institute Inc. 2012).

Figure 1: White trap containing a *G. mellonella* cadaver for the collection of emerging nematodes.

Dead larvae were removed and placed into white traps (reference the Lacy manual) (Figure 1). White traps were constructed from 45 mm petri plates containing a smaller petri dish lid (35 mm x 10 mm) to act as a platform with a piece of moist filter paper acting as bridge to the surrounding water. The nematodes emerged from the cadaver and entered the water facilitated by the filter paper. Nematodes were harvested once per week over 10 weeks by pipetting from the white traps into 50 mL centrifuge tubes. These nematode stocks were stored at 10° C. To replace old and dying nematode stocks 12 weeks after initial harvesting, nematode populations from the Break Neck Road site were exposed to *G. mellonella* as described above and collected via white traps as previously described.

Transferring target bacteria to nematodes

To determine if the bacterial microflora of the nematodes could be altered, nematodes were exposed *E.coli* HB101 (p6TT1) expressing to a red fluorescent protein bacteria (Singer et al. 2010). Treatment arenas were established which were comprised of plates with *E. coli* (strain HB101), plates with *Paenibacillus* sp. (previously isolated from *P. entomophagus*), and plates without any additional bacteria added. A 2.5 ml aliquot of nematode growth media (NGM) (Carolina Biological) were poured into 45 mm petri plates under sterile conditions. The treatment plates were then streaked with bacteria and incubated at 37° C for 2-3 days (Figure 2). Four replicates were produced per treatment for a total of 12 plates. Approximately 200-400 nematodes in 140-150 μl of liquid were pipetted onto each plate (labeled *E. coli*, *Paenobacilus* sp., and control plates) and stored at 20° C for 2 days before nematode were sampled for mortality and the evidence of acquired fluorescence.

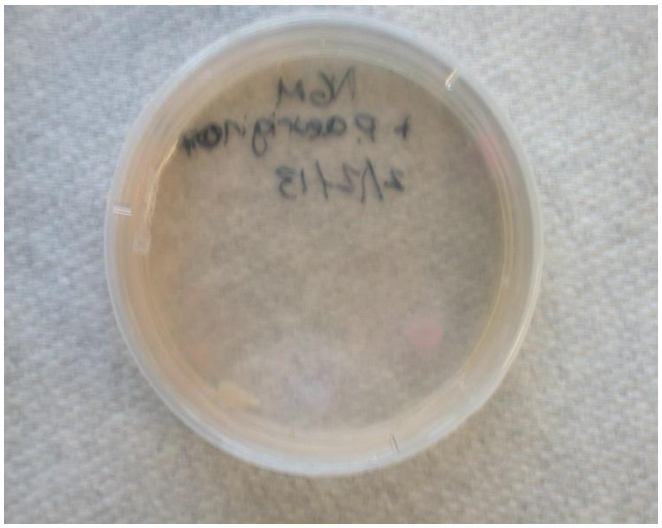

Figure 2: RFP labeled *P. aeruginosa* grown on NGM. NGM is a nutrient-poor media and therefore bacterial growth is not heavy.

To determine mortality, total dead (indicated by straight, stiff or disintegrating nematodes) and living nematodes were counted in the entire plate (*E. coli)* or for plates with high densities of nematodes, a subsample of ca. 50% of the plate. Plates were viewed at 100X magnification under a dissecting microscope for this assessment. Ten to twelve juveniles and ten to twelve adults per plate of both labeled *E. coli* and unlabeled *Paenobacillus* sp. treatments were manually transferred to well slides and viewed on a Zeiss SteREO Discovery.V12 microscope with the Texas Red fluorescent filter (excitation 596/emission 615). The presence of external (cuticle) and internal fluorescence (digestive tract) was determined for adults and juveniles by visual observation (Figure 3). Analysis of variance was conducted to examine the difference in mortality between treatments (*E. coli* trial and *P. aeruginosa* trial) and time (*E. coli* trial only) (JMP, SAS Institute Inc. 2012). Proportion survival was transformed by arcsine square root for analysis (JMP, SAS Institute Inc. 2012)..

This experiment was repeated using a red fluorescent protein (p66TT1 plasmid d-tomato) labeled *Pseudomonas aeruginosa* (strain PA14) instead of the labeled *E. coli*. Nematodes were introduced to bacteria grown on NGM and observed for mortality and presence of fluorescence as described above. Repeated samples were prepared and observations were made every 2-3 days over 13 days to determine changes in the proportion of adult and juvenile nematodes carrying the fluorescent protein labeled bacteria. Repeated measures analysis of variance was used to examine differences between proportion of nematodes showing florescence in the different life stage over time. Proportion florescence was transformed by arcsin squareroot for analysis.

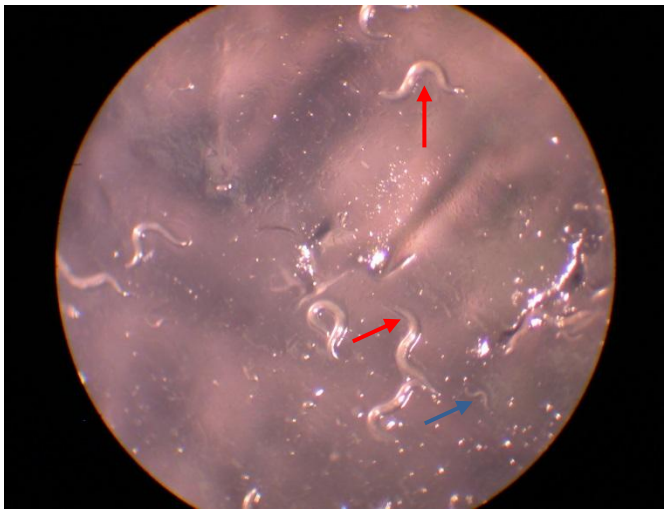

Figure 3: Adult and juvenile nematodes on NGM. Red arrows indicate adults and the blue arrow indicates juveniles.

Transferring RFP labeled bacteria to *Galleria mellonella* larvae via infected nematodes

To determine if nematodes are capable of transferring newly associated bacteria into their insect hosts, waxworms were exposed to nematodes carrying the RFP labeled bacteria. Three different treatments were established: 1) no nematodes, 2) nematodes grown on plates with their naturally associated bacteria, and 3) nematodes grown on plates with RFP labeled bacteria. There were 4 replicates per treatment for a total of 12 plates. Nine *G. mellonella* larvae were placed into each 100×20 mm^2 petri dish containing 20 g of autoclaved sand moistened with 5 ml of autoclaved DI water. Thirty-five live juvenile nematodes where manually transferred from their respective nematode growth agar to the experimental arenas of all treatments using a probe (Figure 4). Nematodes were added to a 50 µl autoclaved DI water droplet within areas cleared of sand. Sand was gently pushed back into place to introduce nematodes into the moist sand. The petri dishes were stored in a humid chamber and waxworms were monitored daily for mortality.

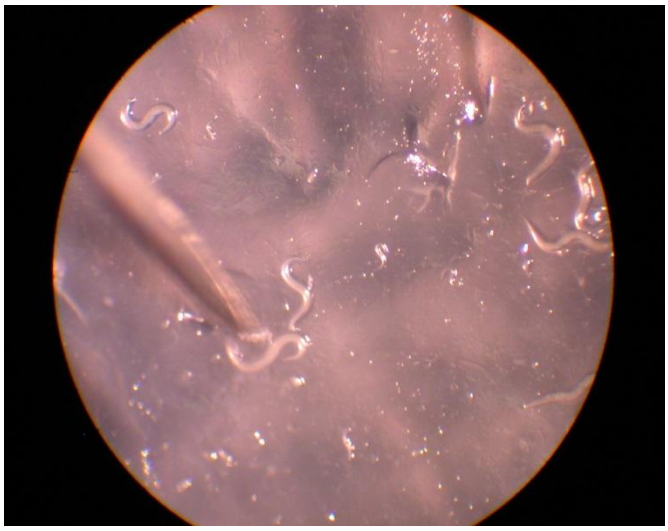

Figure 4: Manual transfer of nematodes. Nematodes are individually picked up using a very fine probe.

Hemolymph from the treated and control *G. mellonella* larvae was sampled prior to death and post death. Larvae were surface sterilized with a 1% sodium hypochlorite solution (the 1% sodium hypochlorite solution used for surface sterilizing was confirmed as non-fluorescing via manual observation using the Zeiss SteREO Discovery V12 microscope.) One to two microlitres of hemolymph were then collected from *G. mellonella* larvae by cutting off one leg with sterile, micro-dissecting scissors, and collecting the welling hemolymph with a 1 μl microcapillary tube. Hemolymph samples were collected from three live larvae from each plate 3 days after introduction of the nematodes. Samples were then collected from each of the remaining waxworms as they died over the eleven day duration of the experiment. Survival analysis was conducted using a general parametric model based on the Weibull distribution to examine difference in time to death between nematode treatments and control (JMP, SAS Institute Inc. 2012).

Hemolymph was added to 20 μl of DI autoclaved water on a standard microscope slide. A 25×25 mm^2 cover slip was placed over the sample and the edges were sealed

with clear nail polish to prevent desiccation. The samples were then viewed at 100X magnification on a Zeiss SteREO Discovery.V12 with the Texas Red fluorescent filter (excitation 543/emission 610) to determine if there was any evidence of fluorescence.

Results

Virulence of Nematode Populations

Nematodes collected from the three different sites had varying levels of virulence when exposed to *Galleria mellonella* larvae. Nematodes collected from Orono (Figure 7) had the highest virulence compared to Visitors Center (Figure 6) with the lowest. Virulence significantly varied by site (X^2=64.91. df=2, 8, p<0.02) and treatment (X^2=64.91, df=2, 8, p<0.0001). Lack of overlap between 95% confidence intervals indicate that larvae exposed to high (200-250) and low (20-25) doses of nematodes from Breakneck Road (Figure 5) and Orono experienced significantly higher mortality compared to treatments without nematodes (Table 2). Overlap of 95% confidence intervals indicates that *G. mellonella* larvae exposed to high and low doses of nematodes from Visitors Center did not have significantly higher mortality compared to those receiving the control treatment with no nematodes. Overlap of 95% confidence intervals indicates there was not a significant difference in mortality between a high and low dose for either Breakneck Road, Orono, or Visitors Center (Table 2).

Table 2: Ninety-five percent confidence intervals from survival analysis of *G. mellonella* larvae after exposure to nematodes from Breakneck Road (BNR), Orono (OR), and Visitors Center (VC) sites.

Treatment	Lower / Upper 95% Confidence Interval
BNR Control	10.67 / 12.50
BNR Low	5.83 / 9.70
BNR High	7.60 / 10.63
OR Control	7.20 / 12.09
OR Low	3.77 / 6.92
OR High	4.19 / 7.17
VC Control	5.95 / 11.14
VC Low	6.33 / 12.87
VC High	5.36 / 10.27

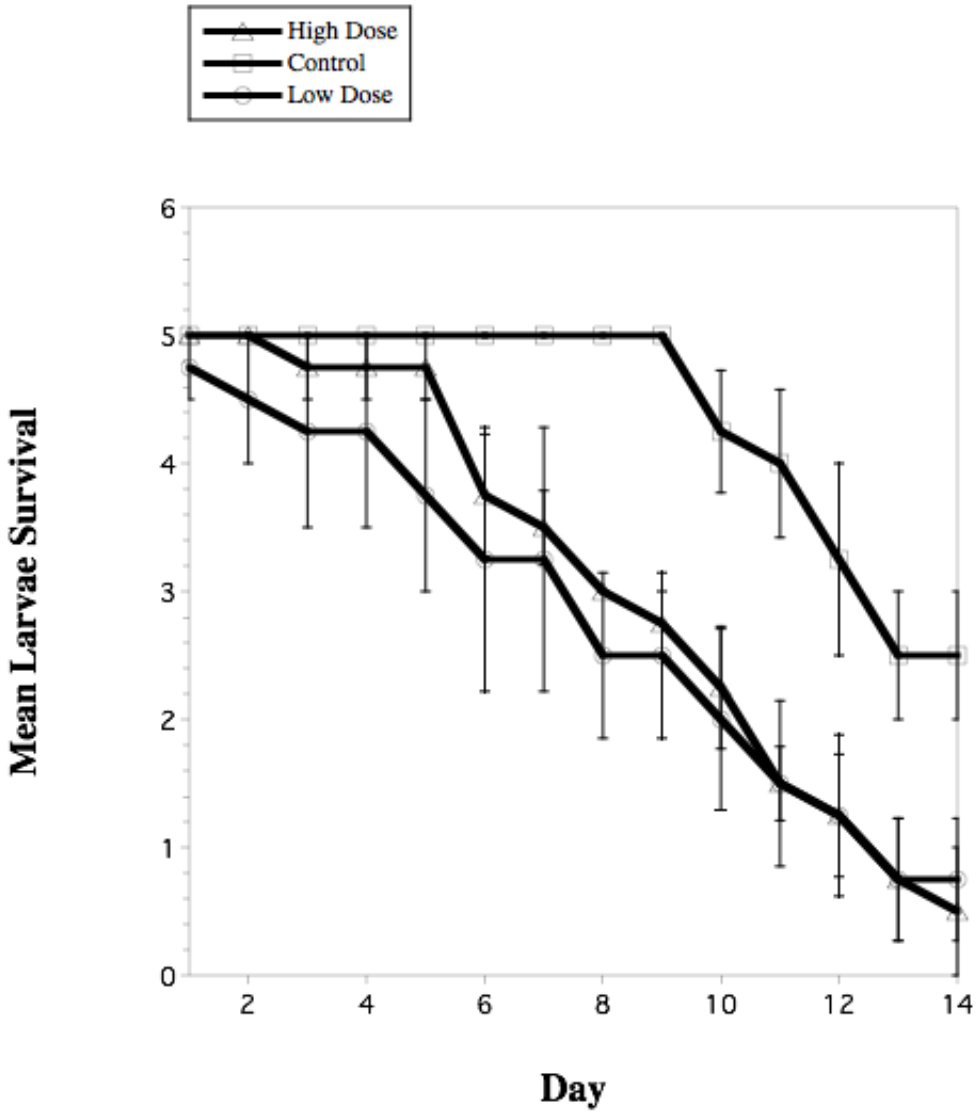

Figure 5. Survival in *G. mellonella* larvae during 14 days of exposure to nematodes that emerged from *M. rubra* colonies collected on Breakneck Road in Acadia National Park, in Bar Harbor, ME. Mean larvae survival indicates the average quantity of waxworms surviving out of the initial five waxworms. (Error bars depict SE)

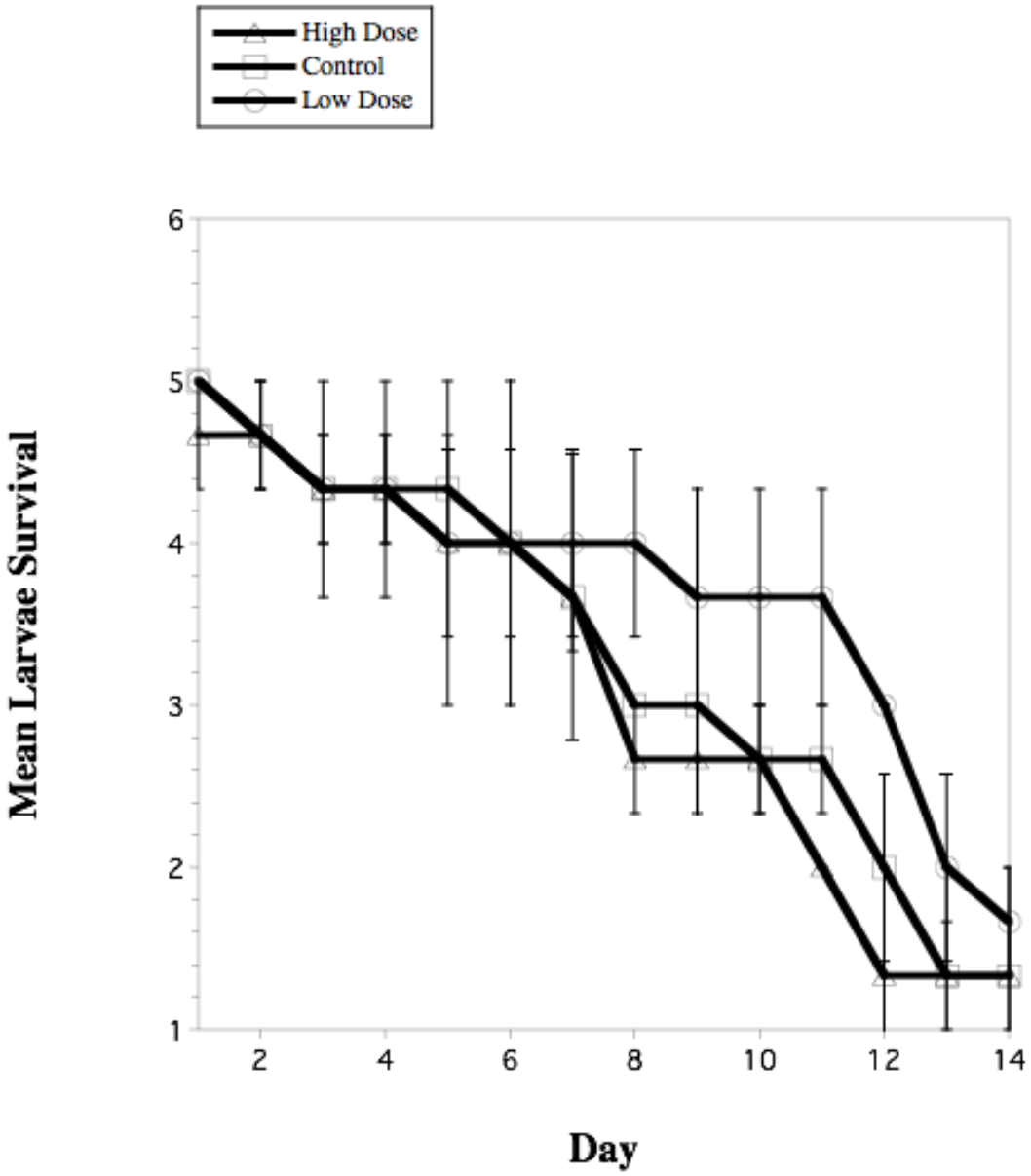

Figure 6. Survival in *G. mellonella* larvae during 14 days of exposure to nematodes that emerged from *M. rubra* colonies collected from the Acadia National Park Visitors Center in Hulls Cove, Bar Harbor, ME. Mean larvae survival indicates the average quantity of waxworms surviving out of the initial five waxworms. (Error bars depict SE)

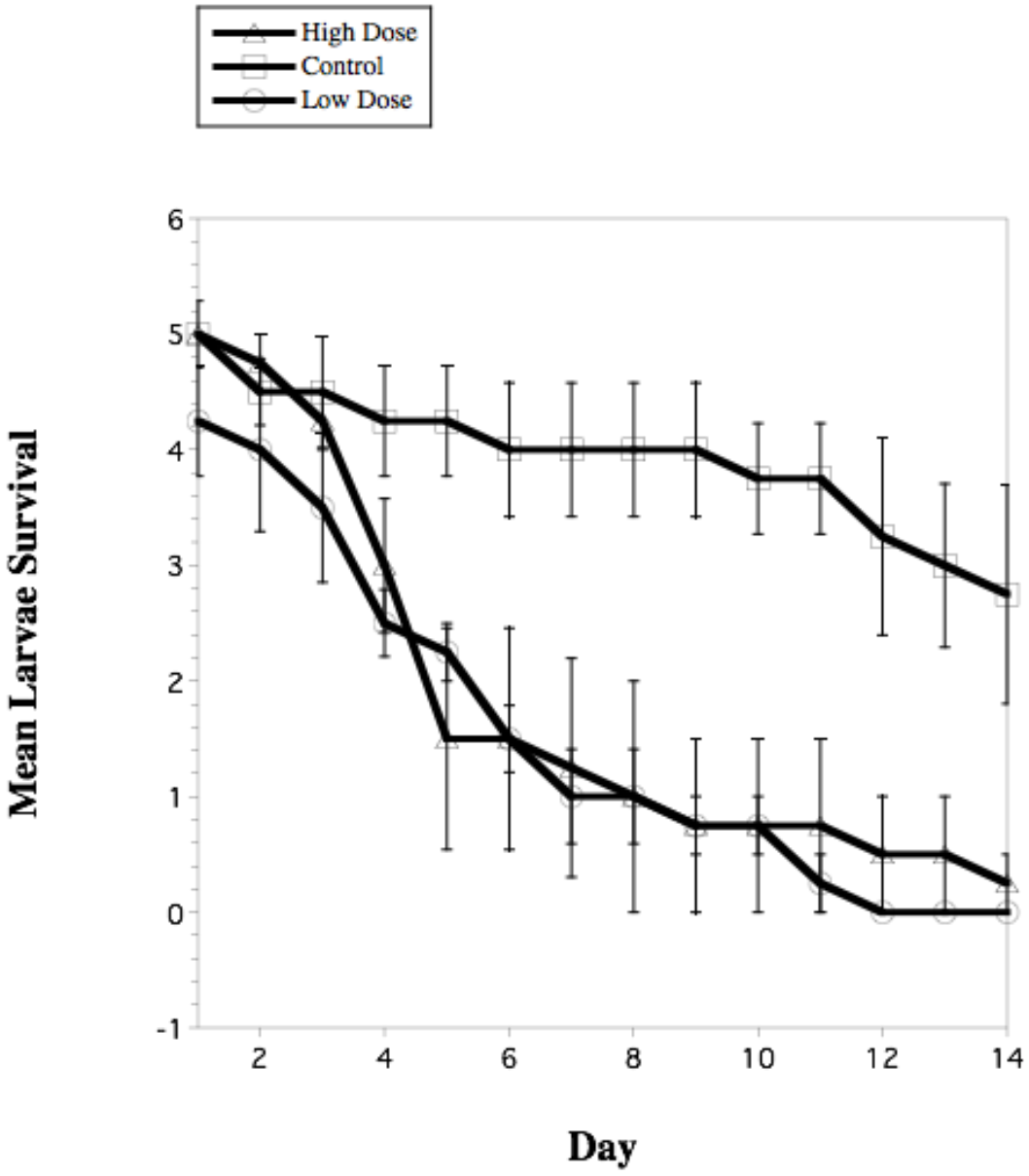

Figure 7: Survival in *G. mellonella* larvae during 14 days of exposure to nematodes that emerged from *M. rubra* colonies collected in Orono, ME. Mean larvae survival indicates the average quantity of waxworms surviving out of the initial five waxworms. (Error bars depict SE)

Nematode Survival after Exposure to RFP Labeled *E. coli* and *P. aeruginosa*

Mortality in nematodes exposed to RFP labeled *E. coli*, *Paenibacillus* sp., or their natural microflora was not significantly different between treatments ($F=0.27$, $df=2, 5$ $p=0.77$). Mortality at day five was significantly higher than mortality at day two ($F=7.08$, $df=1, 6$; $p=0.04$). After 2 days, 87.4 ± 0.06% of nematodes had survived exposure to *E. coli* and after 5 days, 69.4 ±0.2% survived (Table 3).

Mortality in nematodes exposed to RFP labeled *P. aeuginosa* was not significantly different compared to nematodes exposed to their natural microflora after two days of exposure ($t = 0.75$, $DF=7$, $p=0.48$). After 2 days, 44.9 ± 0.07% of nematodes had survived exposure to *P. aeruginosa* compared to 50.8 ± 0.04% survival in nematodes exposed to their natural microflora.

Table 3: Nematode survival after exposure to RFP labeled *E.coli* and *Paenibacillus* sp.after two and five days of exposure.

Treatment	Proportion Survival Day 2 (mean +/- SE)	Proportion Survival Day 5
RFP labeled *E. coli*	0.87 +/- 0.04	0.69 +/- 0.16
Paenibacillus sp.	0.87 +/- 0.001	0.68 +/- 0.05
Control	0.86 +/- 0.004	0.80 +/- 0.003

E. coli Transfer to Nematodes

The transfer of RFP labeled *E. coli* bacteria was confirmed in 5.0 ± 5.7% of live nematodes, and in 0% of dead nematodes (Figure 8). Two out of 40 sampled nematodes had confirmed fluorescent bacteria present. Of the two with fluorescence, both were adult

and had the bacteria located in their digestive tract (Figure 10 D). No fluorescence was detected in the nematodes fed the *Paenobacillus* sp. nor the control nematodes not fed bacteria.

P. aeruginosa Transfer to Nematodes

More nematodes displayed evidence of the fluorescent protein following exposure to the labeled *P. aeruginosa* (Figure 10 A-C) then was observed following exposure to the labeled *E. coli.* The proportion of adults with fluorescence (internal and external) was significantly higher than juveniles, with the exception of day 6 when proportion of juvenile fluorescence was 0.61 ± 0.16 in comparison to adults with 0.43 ± 0.08 (p=.002). The greatest difference in proportion of overall fluorescence and peak of fluorescence in adults was on day 10, with 0.95 ± .06 in adults and 0.30 ± 0.29 in juveniles (Figure 9). There was a significant decrease in the proportion of juveniles with fluorescence (external and internal) over time (p=.038). By day 13 fluorescence dropped in both adult and juveniles, to 0.73 ± 0.22 in adults and 0.16 ± 0.14 in juveniles. Location of fluorescence (internal and external) varied significantly by nematode developmental stage with adults having more internal fluorescence and juveniles with more external fluorescence (p<.0001) and varied over time (p=.0007). External fluorescence in juveniles ranged from 0.56 ± 0.16 (day 6) and 0.05 ± 0.1 (day 13). Internal fluorescence in juveniles was uncommon, and ranged from 0.05± 0.06 (day 6) to 0.12 ± 0.16 (day 13). External fluorescence in adults was rare, and occurred in a single adult nematode on day 10. Internal fluorescence in adults was very common and ranged from 0.95 ± 0.06 (day 10) to 0.43 ± 0.08 (day 6).

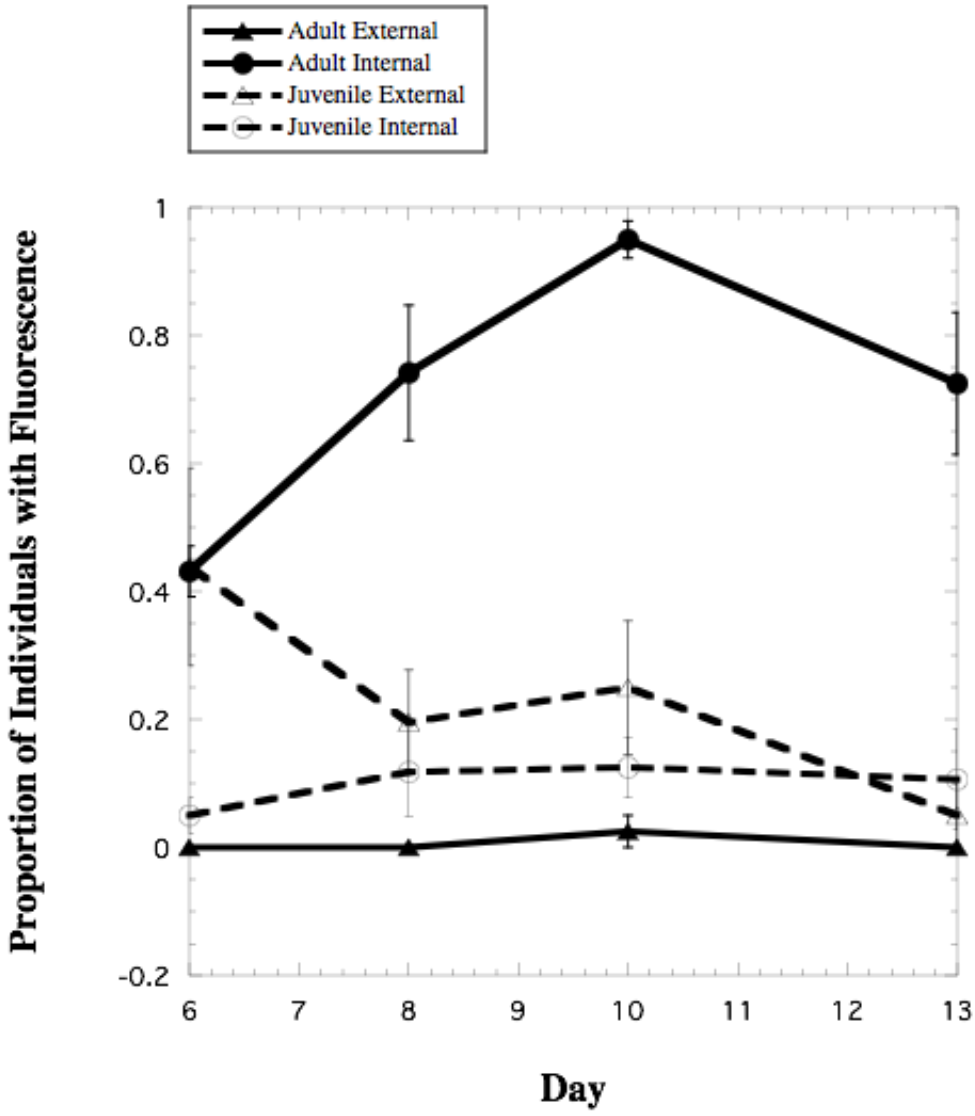

Figure 8: Proportion of adult and juvenile nematodes showing fluorescence from RFP-labeled *P. aeruginosa* either internally in their digestive tract or externally on their cuticle. (Error bars depict SE)

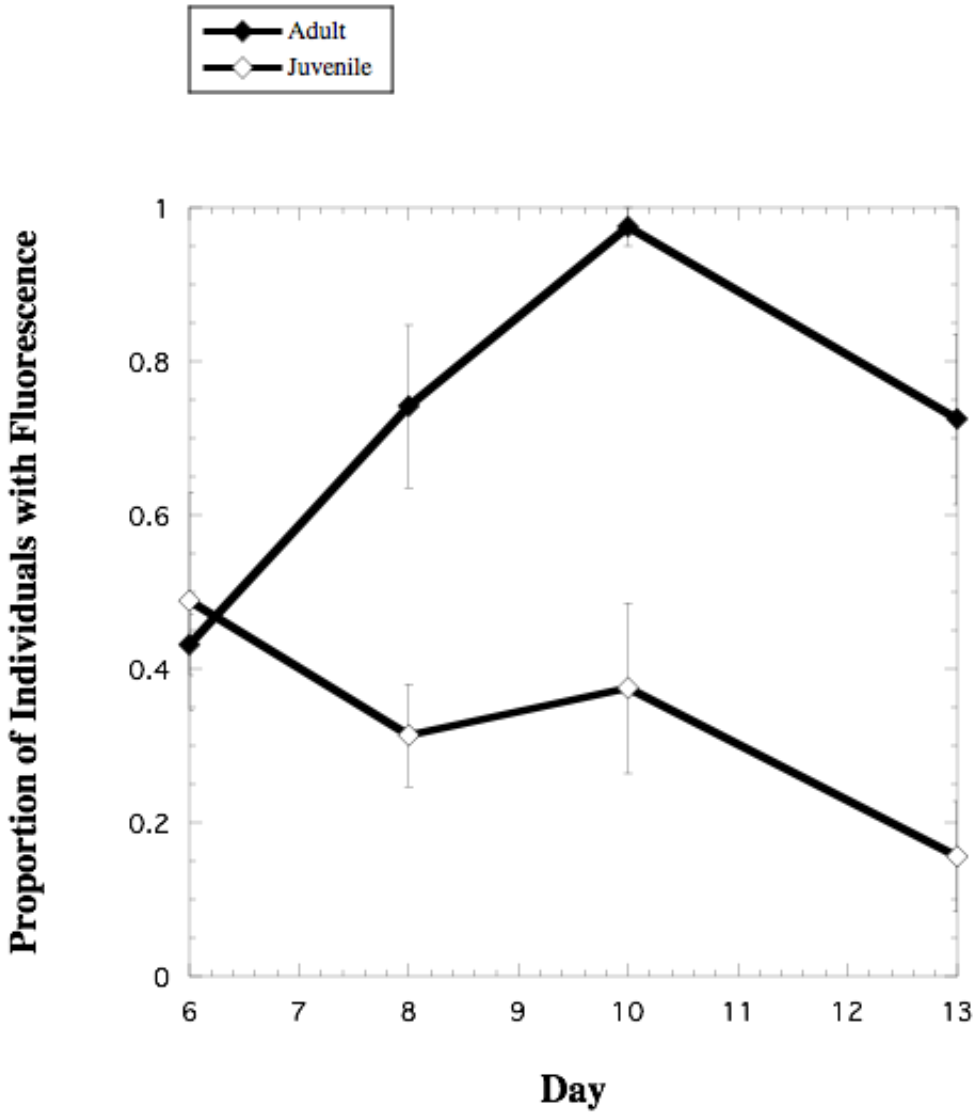

Figure 9: Proportion of adult and juvenile nematodes showing any fluorescence (internal or external) from RFP-labeled *P. aeruginosa*. (Error bars depict SE)

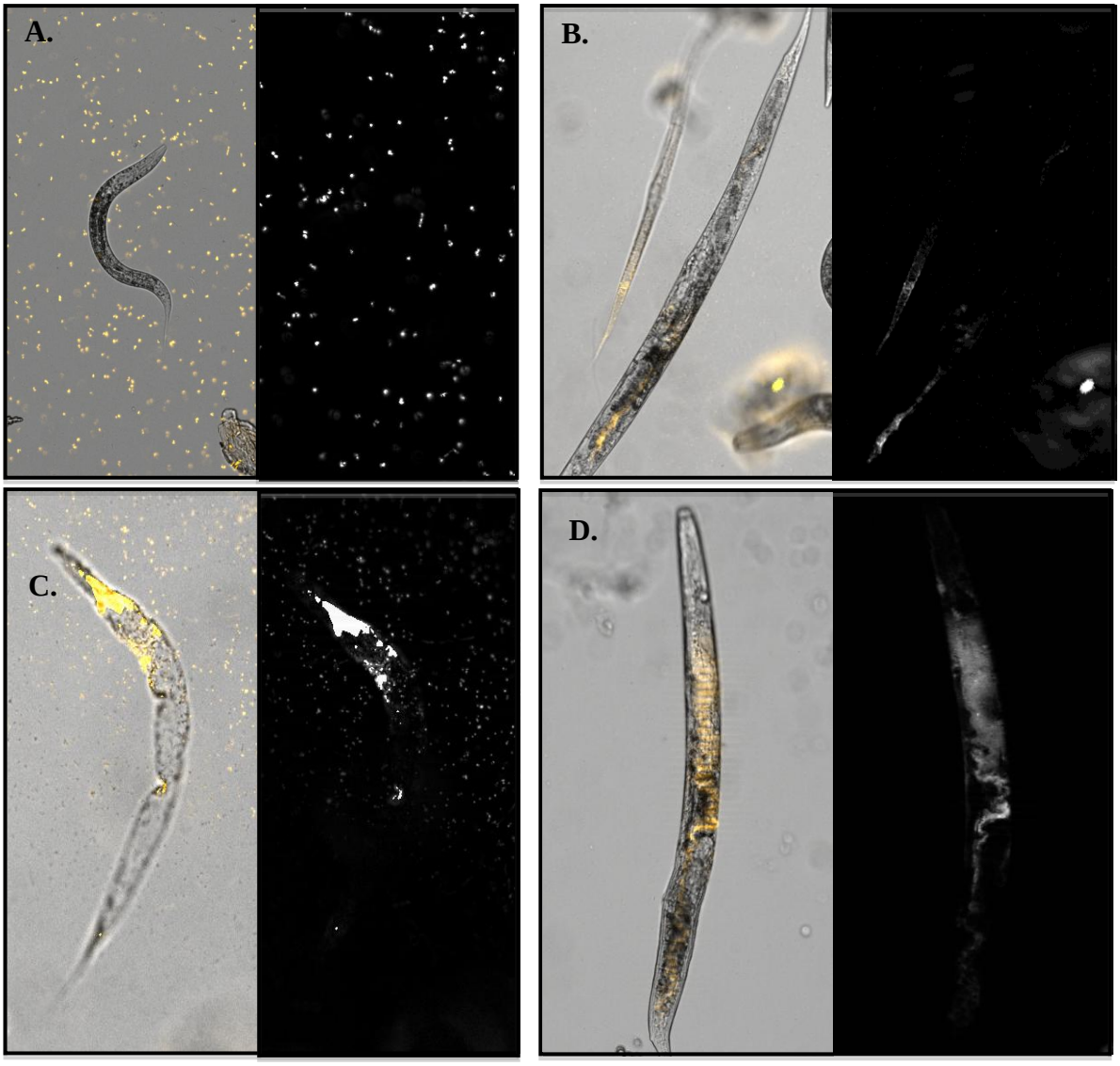

Figure 10: Nematodes exposed to RFP labeled *P. aeruginosa* (A-C) and RFP labeled *E. coli* (D).The images on the left are brightfield with fluorescent microscopy and the images on the right are fluorescent microscopy only. A: juvenile nematode without internal or external fluorescence; C: juvenile nematode with external fluorescence; B and D: adult nematode with internal fluorescence.

Transfer of *P. aeruginosa* to *G. mellonella* Larvae via Nematodes and Larvae Mortality

Over an 11 day period, mortality in *G. mellonella* larvae exposed to both nematodes fed on the RFP-labeled *P. aeruginosa* (X^2=53.32, df=1, 11, p<0.0001) and nematodes reared on nematode growth agar without the bacteria (X^2=65.77, df=1, 11, p<0.0001) greatly increased compared to larvae not exposed to nematodes (control) (Figure 11). Larvae exposed to altered nematodes did not demonstrate significantly higher mortality compared to larvae exposed to unaltered nematodes (X^2=1.92, df=1, 11, p=0.17). By day 11, 96% of larvae exposed to unaltered nematodes had died; 92% of larvae exposed to altered (RFP exposed) nematodes were dead, and 36% of the control larvae were dead. Microscopic observations did not reveal presence of RFP labeled *P. aeruginosa* in hemolymph from any of the waxworm samples.

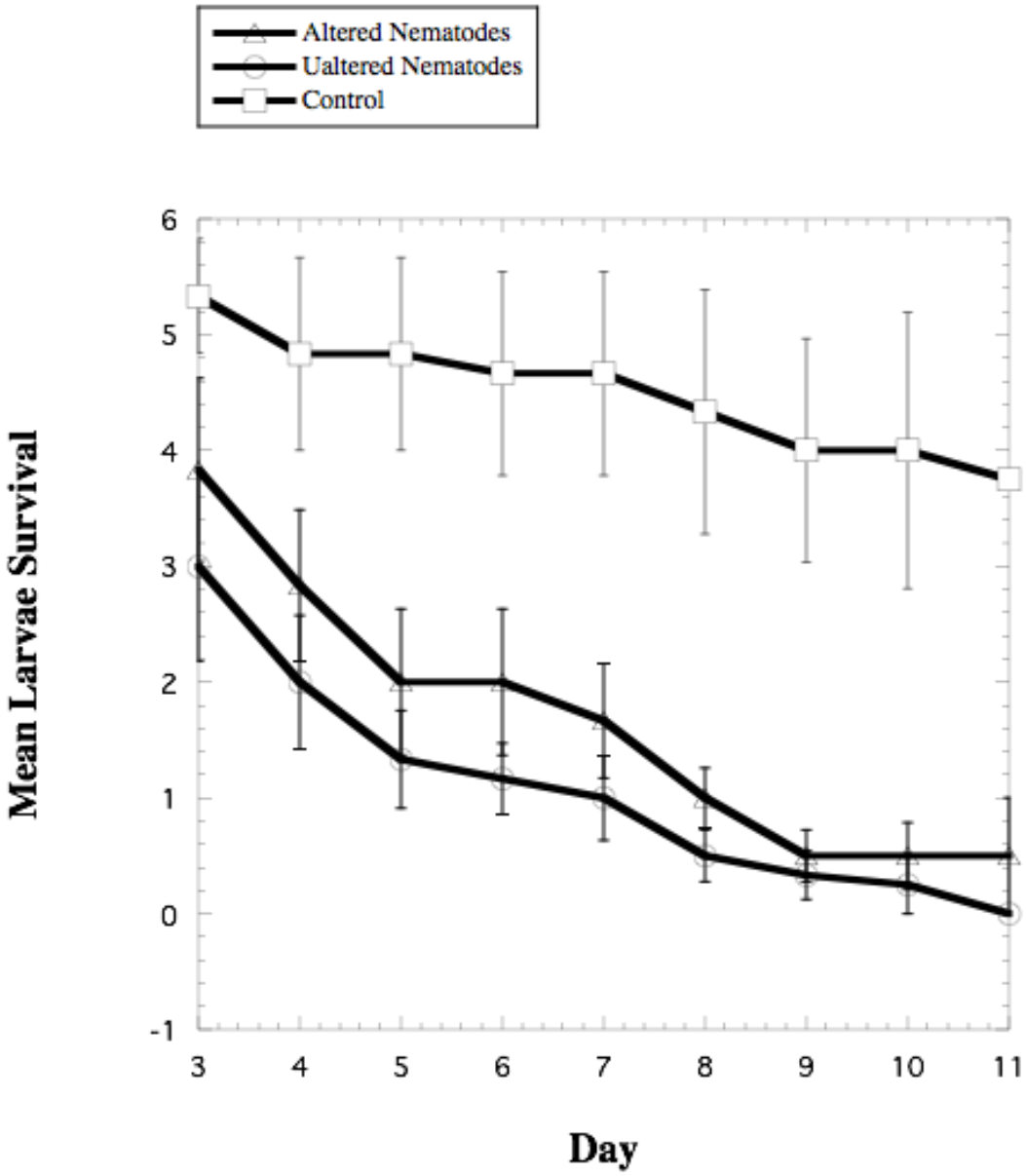

Figure 11: Survival of *G. mellonella* larvae exposed to altered and unaltered nematodes from the Breakneck Road site in Acadia National Park, Bar Harbor, ME. Altered nematodes were exposed to RFP-labeled *P. aeruginosa.* Mean larvae survival indicates the average quantity of waxworms surviving out of the initial six waxworms (Error bars depict SE)

Discussion

Preliminary testing of *P. entomophagus* from different sites indicated variable virulence in *M. rubra* (Groden and Stock 2010). Three sites tested in this project demonstrated variable mortality in *G. mellonella* larvae with only two of the three sites causing significant mortality. These data suggest that differences may exist between *P. entomophagus* populations from different sites, with one hypothesis being that bacterial associates of *P. entomophagus* are variable between sites, and these associated bacteria contribute to insect mortality.

Mesodiplogaster lheritieri (Diplogasterida: Diplogasteridae), a nematode species in the same family as *P. entomophagus*, directly caused mortality by introducing pathogenic bacteria to the insect host's hemocoel resulting in septicemia (Poinar 1969). The types of bacteria isolated from samples of the internal and external microflora of *P. entomophagus* are found in association with other nematodes, insects, fungi, the rhizosphere of plants, and soil (Dumont 2011). Despite an absence of the specific symbiotic bacteria association found in the highly evolved species entompathogenic nematodes, *P. entomophagus* causes mortality in insect hosts including *G. mellonella*, and *M. rubra* (Dumont 2011). Dumont (2011) found bacterial species with documented insecticidal activity associated on the external surface of nematodes. Pathogenic bacteria associated with *P. entomophagus*, are likely picked up from the soil environment and transferred from the internal and/or external surface to the hemocoel of the insect host.

Bacterial isolates from the hemolymph of *P. entomophagus* infected insects include; *Serratia maracescens*, *Serratia nematodiphila*, and *Pseudomonas fluorescens*. *Serratia marcescens*, demonstrated highly insecticidal activity when exposed to

cutworms (Nishiwaki et al. 2007). *Serratia nematodiphila* has been found associated with the entomopathogenic nematode *Heterorhabditidoides chongmingensis* (Rhabditida: Rhabditidae Zhang) (Zhang et al 2009) and was highly virulent in *G. mellonella* larvae (Zhang et al. 2008). *Pseudomonas fluorescens* produces hydrogen cyanide and can cause insect mortality by inhibiting cytochrome *C* oxidase in the respiratory chain of the termite *Odontotermes obesus* (Devi and Kothamsi 2009; Rae et al. 2008). *Proteus vulgaris*, found in *P. entomophagus* collected from Germany, is also pathogenic in insects (Rae et. al 2008; Jander et al. 2000).

Two bacteria, RFP labeled *E. coli* and RFP labeled *P. aeruginosa*, were utilized in the development of protocol to transfer target bacteria to *P. entomophagus*. Strain HB101 of *E. coli* does not have documented virulence in nematodes, and did not cause significant mortality in *P. entomophagus*. The same strain of *P. aeruginosa* (PA14) has demonstrated high virulence in *C. elegans* and *G. mellonella* larvae with an LD_{50} of less than 10 bacteria (Jander et al. 2000). Strain PA14 of *P. aeruginosa* did not cause significant mortality in *P. entomophagus* despite being highly virulent in *C. elegans*. *Pristionchus pacificus*, a closely related nematode, has an abundance of genes encoding for detoxification and degradation enzymes, which is consistent with necromenic and facultative parasitic nematode-insect interactions (Dieterich et al. 2008). This study did not look at other possible effects of pathogenic bacteria on nematodes, such as reduced fecundity (Rae et al. 2008). However, the relatedness of *P. entomophagus* to *P. pacificus* and its resistance to *P. aeruginosa* virulence suggests that *P. entomophagus* may be resistant to many soil pathogenic bacteria.

I was unable to determine if *P. aeruginosa* contributed to *G. mellonella* larvae

mortality as I could not confirm transfer to the larvae. However, the high virulence of strain PA14 in *G. mellonella*, *Drosphila melanogaster* (Common Fruit Fly (Diptera: Drosophilidae Meigen)), and *Pluetella xylostella* (Diamondback Moth (Lepitodoptera: Plutellidae L.)) indicates it would have likely contributed to mortality if transfer was successful. *Galleria mellonella* larvae exposed to nematodes with *P. aeruginosa* on the external surface did not demonstrate significantly higher mortality than unaltered nematodes, further suggesting that transfer did not occur. Nonetheless, *P. aeruginosa* is a potential candidate to alter the microflora and increase virulence of *P. entomophagus* for use as a biological control agent against insects (Jander et al. 2000).

An interesting result was the retention of virulence in *P. entomophagus* from Breakneck Road after cycling through waxworms. Breakneck road nematodes caused significant mortality before cycling through *G.mellonella* larvae. The nematodes isolates exposed to *P. aeruginosa* had not directly been exposed to soil, unlike the nematodes harvested from *M. rubra* cadavers. In a study by Rae et al. (2008), *Pristionchus entomophagus* was significantly more attracted to specific soil and beetle derived bacteria than *E. coli* OP50, an artificial *E. coli* strain used in laboratory culturing of *Pristionchus* nematodes. Chemoattractive profiles may be similar between generations of *P. entomophagus*. If progeny seeks beetle and soil derived bacteria introduced by previous generations, they may be attracted to, consume, and carry pathogenic bacteria even without direct exposure to soil. Further research is needed to determine the retention and changes in virulence between generations of *P. entomophagus*, particularly after stocks are regenerated in *G. mellonella* larvae.

The microflora of adult and juvenile *P. entomophagus* was rarely altered after

exposure to RFP labeled *E.coli*. The absence of fluorescent bacteria in the water surrounding the sample nematodes corroborated the rarity of *E. coli* transfer to the nematodes. The microflora of adult and juvenile *P. entomophagus* was frequently altered after exposure to *P. aeruginosa*. The presence of fluorescent bacteria in the water surrounding the sampled nematodes exposed to *P. aeruginosa* corroborated the high proportion of fluorescent bacteria on the cuticle and in the digestive tract. If the nematodes were frequently consuming the RFP labeled *E. coli*, the presence of RFP labeled *E.coli* in the water surrounding the nematodes would be expected as a result of defecation of consumed bacteria. RFP labeled *E. coli* is not an ideal bacteria for altering the microflora of *P. entomophagus* as it is likely infrequently consumed by nematodes and does not readily adhere to the cuticle. *Escherichia coli* OP50 is used in laboratory cultures of *C. elegans* and *P. pacificus* as it has limited growth on NGM which allows for ease of observation and facilitates mating of the worms (Stiernagle 2006). While *P. pacificus* appears to have consumed *E. coli* OP50 when it was the only bacteria provided, it consistently preferred soil-derived bacteria to *E. coli* (Rae et al. 2008). As *E. coli* HB101 is not frequently used in nematode culture, little research exists to indicate if it is suitable as a nutritional source for *Pristionchus* nematodes.

The internal microflora of most adult nematodes was altered after exposure to *P. aeruginosa*, as adults readily consume *P. aeruginosa*. *Pristionchus* nematodes in chemotaxis assays were attracted to *Pseudomonas* strains to varying degrees (Rae et al. 2008). However, the internal microflora of juvenile nematodes was rarely altered as juveniles appear to seldom consume *P. aeruginosa*. A potential explanation for alteration of external microflora occurring almost exclusively in juveniles is the retention of the

second instar cuticle in third instar daeur juveniles (Bellows 1999). The RFP labeled *P. aeruginosa* may be contained between the two cuticle layers rather than on the external surface, and potentially would remain between the cuticles even during sampling. The lack of evidence of the labeled bacteria in the cuticular surface of the adults may be explained by *P. aeruginosa* being easily washed off of the external surface during sampling, as adults have only one cuticle. Enhancement of virulence in juvenile nematodes is necessary for enhanced virulence in insect hosts. Juveniles are the infective stage, and without internal haborage, they may be poor vectors of the target bacteria. Juveniles exposed to the labeled bacteria were seen to frequently carry it on the external surface of their cuticle. Age-dependent differential dietary preference may influence which bacterial species can alter the nematode's microflora.

The nematode life stages did exhibit different trends over time. In juveniles, the labeled bacteria were almost always found adhered to the external surface of the cuticle. In adults, the labeled bacteria were almost always found in the digestive tract. Over time the quantity of adult nematodes with fluorescence increased and the quantity of juveniles with fluorescence decreased. Further research is needed to determine the length of viability of enhanced virulence in *P. entomophagus*. My results demonstrated the presence of the RFP labeled *P. aeruginosa* on the cuticle of juveniles for at least 13 days. However this only indicates the presence of the bacteria, not necessarily the viability.

Another interesting question is the viability of bacteria consumed and defecated by *P. entomophagus*. A study found that *Pristionchus lheritieri* exposed to *S. marcescens, A. tumafaciens, E. amylovara, E. carotovora,* and *P. phaseolicola* carried viable bacteria for up to 30 hours (Chantanao and Jensen, 1968). Current research does not indicate the

presence of a bacterial receptacle or special morphological structure for bacterial dissemination in *Pristionchus entomophagus,* unlike the entomopathogenic nematode families such as Steinernematidae and Heterorhabditidae which do have a specialized bacterial receptacle for maintaining their symbionts (Snyder et al. 2007). Dissemination of bacteria from *P. entomophagus* likely occurs through defection and facultative transfer from the contaminated external cuticle. Diplogasterid nematodes lack a grinder in the terminal bulb, and therefore are able to disseminate intact bacteria (Poinar 1975). However, the proportion of bacteria that remain intact is unknown, as some bacteria are lysed and digested (Rae et al. 2008).

I was unable to transfer RFP labeled *P. aeruginosa* to *G. mellonella* larvae. There are many potential causes, including; inability of *P. aeruginosa* to compete with other bacteria present in the cadaver, insufficient conditions for *P. aeruginosa* growth, poor viability of transferred bacteria, or most likely, insufficient bacteria present on the cuticle of juveniles. Fluorescent microscopy suggests that the presence of *P. aeruginosa* in the digestive tract in juveniles was rare or present in relatively low quantities, and transfer relied upon the presence of the bacteria on the external surface. If the labeled bacteria was present between the second and third instar cuticles rather than external surface, it may not be able to transfer to the insect host due to its location between cuticles. Altered nematodes were added to moist sand, where *P. aeruginosa* may have been washed off or mechanically removed as the nematodes moved through the sand. Previous studies suggest that *P. aeruginosa* strain PA14 is highly virulent in *G. mellonella* larvae (Jander et al. 2000). If transfer had occurred, higher mortality would likely occur in treatments with altered nematodes. In this study, altered nematodes did not demonstrate significantly

higher mortality than unaltered nematodes exposed to *P. aeruginosa*, suggesting that *P. aeruginosa* did not transfer to the larvae.

Overall, I was able to develop a protocol to transfer *P. aeruginosa* to *P. entomophagus* resulting in alteration of the microflora. Further research is necessary to develop a protocol to transfer a target bacteria carried by *P. entomophagus* to an insect host. Transfer by nematodes carrying the target bacteria externally may be possible, but potentially is less likely to transfer. Any potential bacterial species utilized in alteration of the microflora should be readily consumed by both juveniles and adults and should not cause significant mortality or reduced fecundity. *P. entomophagus* is likely resistant to many soil-derived pathogens, making a variety of pathogenic bacteria potential targets for enhanced virulence and use a biological control agent.

Conclusions and Future Directions of Study

Similar research into the alteration of microflora in necromenic and facultative Diplogasterid nematodes to enhance virulence has not been conducted. I was able to develop a protocol for the alteration of the microflora of *P. entomophagus*. However, I was unable to confirm transfer of the target bacteria by nematodes to an insect host, *G. mellonella* larvae. Nonetheless, this project demonstrated the feasibility of using *P. entomophagus* with enhanced virulence as a biological control agent against European fire ants and potentially other insect pests. However, because of how new this area of research is, it is currently only at a preliminary stage.

The first challenge in developing *P. entomophagus* as a biological control agent is the need for further research on *Pristionchus entomophagus* in general, as little research focused upon *P. entomophagus* currently exists. Most research within the genera is focused upon *P. pacificus* as a comparative organism to *C. elegans* and as a model organism for developmental and evolutionary biology (Mayer et al. 2007; Hong 2006). Other research on *Pristionchus* nematodes has focused upon phylogeny and diversity of the genus (Mayer et. al. 2007). The current research on *P. entomophagus* includes several insect-nematode associations, chemosensory responses, geographic distribution, and preliminary identification of the microflora.

An important area of research in understanding the virulence mechanisms of *P. entomophagus* is the identification and origins of the microflora, mechanisms of transfer to the insect host, and the bacterial species that contribute to host mortality. Initial research on bacteria associated with *P. entomophagus* identified four bacterial isolates from the hemolymph of infected hosts that may contribute to host mortality. Though

three of these isolates have documented insecticidal activity, further research is needed to verify the link between bacterial isolates found in *P. entomophagus* microflora and insect mortality (Dumont 2011).

An important research question is determining which pathogenic bacterial species could be used to enhance virulence. A pathogenic bacterial species that causes significant mortality or reduced fecundity is not an ideal candidate. The microflora of less virulent *P. entomophagus* strains could be altered with insecticidal bacteria associated with more virulent strains of *P. entomophagus*. Less virulent *P. entomophagus* strains may lack insecticidal bacteria associated with more virulent populations because specific bacterial species may be rare or absent in the soil microflora they originated from. The presence of a specific pathogenic bacterial species in more virulent *P. entomophagus* may indicate that is less likely to cause significant mortality or reduced fecundity in less virulent *P. entomophagus*.

The microflora of *P. entomophagus* could also be altered with insect pathogenic bacteria not typically found in association with nematodes that associate with insects or typically found in soil microflora. *Pseudomonas aeruginosa* has not been found in nematodes that infect insects. The strain of *P. aeruginosa* utilized in this study, PA14, is highly virulent to a variety of organisms, including the related nematode *C. elegans*. I did not find that this strain of *P. aeruginosa* caused significant mortality in *P. entomophagus*, making it a potential candidate to increase the virulence of the microflora. I did not look at another possible impact of pathogenic bacteria, reduced fecundity and reduced brood size (Rae et al 2008). *Pristionchus entomophagus* has a greater resistance than *C. elegans* to pathogenic bacteria, as it has evolved various mechanisms to survive

in the harsh and toxic environment of insect cadavers (Rae et al. 2008). Some mammalian pathogens including; *Proteus vulgaris*, *Proteus mirabilis*, and *Serratia marcescens*, can also infect insects and may also be viable candidates to increase the virulence of P. entomophagus microflora (Chadwick et. al. 1990). In chemotaxis assays, *P. entomophagus* was strongly attracted to *P. vulgaris* and moderately attracted to *S. marcescens* (Rae et al. 2008), making these pathogens promising candidates for future study. A potential issue with using non soil derived pathogenic bacteria to alter the microflora is the specific chemoattraction profiles of *P. entomophagus*. *Pristionchus entomophagus* may be less likely to be attracted to bacteria that is not naturally found in the soil or beetle microflora and therefore may not consume it. Another potential issue is the ecological implications of utilizing pathogenic bacteria not typically associated with soil microflora. Introducing non-soil pathogenic bacteria to the soil may have unintended consequences on the soil ecosystem. Further studies are needed to determine the tolerance and chemoattraction of *P. entomophagus* to insect-pathogenic bacteria and the ecological implications of introducing new bacterial strains and species to the soil ecosystem.

The mechanism of bacterial transfer to insect hosts is unclear in *P. entomophagus* and is essential to understanding how the pathogenic bacterial species within the microflora affects virulence. It is unknown if the location of pathogenic bacteria (internal or external) on *P. entomophagus* influences insect mortality. A previous study found *Pseudomonas fluorescens* on the external surface of *P. entomophagus* and the hemolymph of infected *G. mellonella* larvae, suggesting transfer may occur from the external surface to insect host (Dumont 2011). However, further research is needed to

confirm the link between external harborage of pathogenic bacteria and transfer to insect.

The lack of transfer to the insect host in this project may indicate the importance of internal harborage, as bacteria adhered to the external surface may be a more transient association than internal harborage. Unlike the more familiar nematode genera used for biological control agents, Steinernematidae and Heterorhabditidae; *Pristionchus* nematodes lack specialized morphological features for harboring and disseminating pathogenic bacteria in an insect host (Rae et. al.; Snyder et al. 2007). Insect mortality from diplogasterid nematodes through introduction of associated bacteria and subsequent septicemia has been documented with *M. lheritieri* (Poinar 1969). Dissemination likely occurs through ingestion and defection of intact bacteria (Chantanao and Jensen 1968) and potentially transfer of bacteria adhered to the cuticle or the hemolymph (Dumont 2011). Further study is needed to understand the importance of the location of bacterial associations in bacterial dissemination in *Pristionchus entomophagus.*

If location of pathogenic microflora is essential for enhanced virulence, then dietary preferences may be a limitation. The absence of RFP labeled *P. aeruginosa* in the digestive tract of juveniles and high prevalence in the digestive tract of adults potentially indicates differential nutritional preferences between adults and juveniles. This provides a potential explanation for the decrease in juveniles with external fluorescence over time, as less bacteria would be present to adhere to the cuticle as adults consume it. The putative heavy consumption by adults and lack of consumption by juveniles, poses a challenge to enhancing the virulence of *P. entomophagus* juveniles with *P. aeruginosa* and potentially other insect pathogens. The results of this research potentially indicate that if juveniles do not actively consume the selected pathogen, they may be less likely to

transfer it to the insect. Currently no research on age-dependent differential nutritional preferences in nematodes is available. Further research is needed to determine the nutritional preferences in juvenile and adult *P. entomophagus*, and how the different preferences may impact the location and efficacy of microflora alteration.

I was unable to confirm transfer of the target bacteria by nematodes to *G. mellonella* larvae. This was not surprising as I was only able to run a single experiment. It is most likely that *P. aeruginosa* did not transfer, as altered nematodes did not demonstrate increased mortality in *G. mellonella* larvae as would be expected from this strain of *P. aeruginosa* (Jander et. al. 2000). For the purpose of confirming bacterial transfer from *P. entomophagus* to an insect host, nematodes could be pipetted directly on the dorsal cuticle of *G. mellonella* larvae (Dumont 2010). This technique would be impractical for use of nematodes as a biological control agent. However, it would determine if the target bacteria was transferred from the nematode to the host and if it proliferated within the insect host. Techniques for field application of *P. entomophagus* were not considered in this project.

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Appendix A: Sugar Diet for *Myrmica rubra*

Materials:

- 60g sugar
- 4.5g Tic agar
- 235.5mL water (distilled)

Directions:

1. In a 500ml flask, *gently* insert a stir bar, and pour in about a quarter of the distilled water
2. Place the flask on a hot plate, and turn the hot plate to a low heat, and the stirring setting to a medium speed
3. Measure out 60g of sugar and add it to the flask (using a funnel if needed)
4. Measure 4.5g of agar, and then *slowly* add the agar to the flask
5. Add remaining water to the flask, allowing the water to run down the sides to catch any agar or sugar left on the sides. The heat can be changed to a higher setting (4 is a good level) and the stir speed may be increased as well
6. Watch the flask carefully because we don't want it to boil, and allow the solution to mix on the hot plate until the agar and sugar are completely dissolved (85C minimum, 130C maximum). If the solution starts to boil before the agar and sugar dissolves, turn the heat down and keep the spin speed high.
7. Once the sugar and agar are dissolved, remove the flask from heat using gloves (It's hot!)
8. Carefully pour hot sugar diet into CLEAN Petri dishes (makes about 12) pouring in just enough to completely cover the bottom of the dish
9. Allow to cool, then covers may be placed on the Petri dishes
10. Wrap lids with Parafilm

Appendix B: Tuna Diet for *Myrmica rubra*

Hazards:

Heat: the diet needs to be stirred and heated to temperatures close to 100° C

PPE:

Lab coat, gloves (latex or alternative), heat resistant gloves or mittens

Materials:

- Blender
- Heat plate
- Stirring bar
- Stir stick (something to stir the solution manually)
- Thermometer
- 500 ml flask
- 110 ml distilled water
- 3 g colloid
- 86 g Tuna (StarKist Chuklight tuna in water)

Directions:

1. Measure out 110 ml of distilled water, 86 g of tuna, and 3 g of colloid.
2. In a blender, add 110 ml of distilled water and 86 g of tuna and blend for at least one minute.
3. With out turning the blender off, slowly add the 3 g of colloid.
4. Blend for at least a minute, and the solution should look gooey.
5. When the solution looks gooey, turn off the blender and transfer the solution to the 500 ml flask.
6. Place the flask on the heat plate, on a heat setting of 4, and stir the solution manually.
7. Once the solution becomes liquid, a stirring rod may be added at a medium speed, and the temperature can be turned to a heat setting of 5 or 6.
8. As the solution becomes more liquid and less gooey, check the temperature. The solution is done when the temperature has reached at least 80° C (176° F) but do not allow the temperature to exceed 130° C (248° F).
9. At this point the solution should be very liquidy and easy to use. Turning off the heat plate and wearing heat resistant gloves, carefully pour the solution into petre dishes.
10. Allow them to cool, then covers can be added.

If the solution does not solidify, it is possible that:

1. There was not enough heat during the process
2. There was not enough colloid in the solution. The solution should always be 1.5% w/w for a solid but still gelatinous substance. A higher percentage can be added for a harder solution, less for a softer solution.

Author's Biography

Amy M. Michaud was born in Caribou, Maine on February 20, 1990. She was raised in Westfield, Maine and graduated from Presque Isle High School in 2008. Majoring in biology, Amy has a minor in chemistry. She studied two semesters abroad at University of East Anglia in Norwich, England. She received a Gilman Scholarship and Center for Undergraduate Research Fellowship.

Upon graduation, Amy plans to return to England and travel before pursuing an advanced degree in parasitology, public health or infectious disease.